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**Innate Immunity in Stem Cell-Derived Hepatocytes**

- Review -

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**Abstract**

Stem cell-derived hepatocyte-like cells (HLCs) offer great opportunities for studies of host-pathogen interactions and tissue regeneration, as well as hepatotoxicity. To reliably predict the outcome of infection or to enhance graft survival, a finely tuned innate immune system is essential. Hepatocytes have long been considered solely metabolic and their critical innate immune potential is only recently gaining attention. Viral infection studies show that pathogen detection by cytosolic receptors leads to interferon (IFN) induction in primary hepatocytes and HLCs. IFN expression in HLCs is characterised by strong expression of type III IFN and low expression of type I IFN which is also a characteristic of primary hepatocytes. The response to IFN differs in HLCs with lower interferon-stimulated gene (ISG)-expression levels than in primary hepatocytes. TNF- $\alpha$  signalling is less studied in HLCs, but appears to be functional. Expression of toll-like receptors (TLR) 2-5, 7 and 9 have been reported in primary hepatocytes but have been poorly studied in HLCs. In summary, although they retain some immature features, HLCs are in many ways superior to hepatoma cell lines for cell based modelling. In this review, we will provide an overview of innate immune signalling in HLCs and how this compares to primary hepatocytes.

**Key words:** Stem cells, hepatocytes, innate immunity

**1. Introduction**

Stem cell-derived hepatocyte-like cells (HLCs) are of interest as alternatives to primary cells for the study of host-pathogen interactions, tissue regeneration and drug-development. The discovery of somatic cell reprogramming in 2006, which allows the generation of pluripotent stem cells, has revolutionised the field of stem cell research. The so-called induced pluripotent stem cells (iPSCs) can be produced from the desired genetic background providing diverse stem cell banks (1). In contrast, human embryonic stem cells (hESC) are derived from the inner cell mass of preimplantation embryos that are not suitable for human implantation, with the first hESC lines described in 1998 (2). Currently, both hESC and iPSC are used in stem cell research and for differentiation into somatic cells.

The immunogenicity of pluripotent stem cell-derived HLCs and other somatic cells has been studied extensively while the ability of *in vitro* differentiated HLCs to mount an immune response by sensing and reacting to pathogens has only recently gained attention (3-13). As the major metabolic cell type of the liver, hepatocytes are constantly exposed to diverse metabolic products and antigens including the products of digestion as well as hepatotropic pathogens. In this microenvironment the hepatocyte has to be immune tolerant to food- and commensal-derived antigens while staying alert to invading pathogens. To manage the dichotomy between immune tolerance and immune activation, the hepatic innate immune system is tightly regulated. *In vitro* differentiated HLCs that

51 closely mimic primary hepatocytes would be of significant value in identifying these regulatory  
52 mechanisms.

53  
54 Studies of host-pathogen interactions have long relied on hepatoma cell lines where innate immune  
55 responses are impaired and intracellular signalling is not representative of healthy hepatocytes (3, 4,  
56 14-16). HLCs more closely mimic the *in vivo* situation. Moreover, as they possess the genetic  
57 background of their donor, HLCs can be used to study donor specific influences on hepatocyte  
58 function (17). Hence, HLCs are a promising tool for understanding cell-based immune responses to  
59 pathogens and provide a platform for identification of the mechanisms that underlie inter-individual  
60 differences in susceptibility to hepatotropic pathogens.

61  
62 Discovering host genetic factors that determine the outcome of hepatotropic infections and  
63 response to antiviral therapy will also lay the basis for the development of new and better therapies  
64 with fewer side effects. Host factors important for viral entry and replication, viral sensing  
65 mechanisms and the induction of innate immune responses in infected cells all together determine  
66 the outcome of infection. For viruses that establish chronic infection such as hepatitis B (HBV) and  
67 hepatitis C virus (HCV) the effectiveness of the innate immune response mounted by the host cell  
68 determines whether infection becomes chronic or is cleared successfully (18-21). In order to enhance  
69 the hosts' innate immune response and thereby increase the chances of clearing the virus, immune-  
70 based therapies are of continued interest for the treatment of HBV and HCV. At the same time,  
71 these therapies are of benefit for the treatment of viruses with high genetic variation such as HCV  
72 (22).

73  
74 With regard to regenerative medicine, functional innate immune responses in stem cell-derived  
75 somatic cells to be used for transplantation will improve graft survival and allow the cells to react to  
76 and fight off invading pathogens. A tightly regulated immune response is particularly important in  
77 the liver, an organ that is constantly exposed to a wide variety of antigens including both, food-  
78 derived antigens and pathogens (23).

79 This review will provide an overview of our current knowledge of the innate immune machinery of  
80 stem cell-derived HLCs and how it compares to primary hepatocytes. Expression of viral sensing  
81 molecules, downstream signalling pathways and generation of antiviral and pro-inflammatory  
82 cytokines will be discussed in relation to the *in vivo* situation with the aim of better understanding  
83 the immune phenotype of *in vitro* differentiated HLCs.

## 84 85 86 **2. Innate immune pathways in SC-derived hepatocytes**

87 Protein-based innate immune responses, such as virus sensing by pattern recognition receptors  
88 (PRR) and the interferon response are not detectable in pluripotent cells and are only acquired  
89 during maturation (5, 24, 25). Innate immunity in pluripotent stem cells was therefore long thought  
90 to be severely attenuated. However, more recent studies of defense strategies of pluripotent stem  
91 cells suggest RNAi as a major antiviral mechanism (5, 24, 25). In contrast, innate immune strategies  
92 in differentiated cells comprise detection of pathogen-associated molecular patterns (PAMPs),  
93 which are conserved structures within a pathogen, by PRRs followed by expression of IFN and ISGs  
94 and intrinsic antiviral immunity.

95  
96 Despite the progress made in differentiation procedures, HLCs retain an immature phenotype, still  
97 expressing markers of stem cells and fetal hepatocytes (26, 27). It is therefore not farfetched to  
98 assume that innate immune pathways might also be of an immature phenotype in the *in vitro*  
99 differentiated cells. From the few studies that have investigated innate immunity in stem cell-  
100 derived somatic cells, there is some evidence that the IFN-based arm of innate immunity and also  
101 toll-like receptor (TLR)-responses remain down-regulated (figure 2). However, research of these  
102 mechanisms in HLCs is still limited, requiring further studies into the antiviral response. In the

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2  
3 103 following section, we will describe hepatic innate immune mechanisms and their presence or  
4 104 absence in HLCs.  
5 105  
6 106  
7 107 *2.1 Viral sensing by cytosolic PRRs*  
8 108 Hepatocytes are equipped with a variety of cytosolic PRRs that allow them to detect PAMPs from  
9 109 DNA- and RNA- viruses, intracellular bacteria and damage-associated molecular patterns (DAMP),  
10 110 released from damaged cells. Retinoic acid inducible gene-I (RIG-I) and melanoma differentiation-  
11 111 associated gene 5 (Mda-5) are sensors of viral dsRNA. Ligand binding triggers a signalling cascade  
12 112 via mitochondrial antiviral signalling protein (MAVS) and interferon regulatory factor 3 (IRF3)  
13 113 leading to the induction of IFNs. While RIG-I senses short dsRNA motifs, long dsRNA is sensed by  
14 114 Mda-5. Ligand recognition of RIG-I or Mda-5 results in receptor recruitment to the mitochondrial  
15 115 surface where binding to MAVS via the respective CARD domains occurs. The complex of RIG-  
16 116 I/MAVS and Mda-5/MAVS respectively activates TANK-binding kinase 1 (TBK-1) and IκB kinase-ε of  
17 117 the IκB kinase complex which phosphorylate IRF3. Phosphorylated IRF3 translocates to the nucleus  
18 118 and induces the expression of type I and type III IFNs (figure 1) (28).  
19 119 Viral DNA is detected by the cGAS-STING signalling pathway (29, 30). cGAS detects dsDNA and  
20 120 induces STING to recruit TBK-1 and IRF3 to its C-terminus. The close proximity between TBK-1 and  
21 121 IRF3 allows TBK-1 to phosphorylate IRF3 which ultimately leads to IFN-release.  
22 122  
23 123 Infection studies with HCV and HBV revealed that cytoplasmic PRRs in HLCs, including RIG-I and  
24 124 cGAS, sense infection and mount an innate immune response against both RNA and DNA-viruses.  
25 125 The use of polyI:C to mimic dsRNA revealed that HLCs detect HCV-infection through RIG-I, resulting  
26 126 in strong upregulation of type III and weaker upregulation of type I IFNs (3, 6). Late and low level  
27 127 induction of type I IFNs is also characteristic of primary human hepatocytes. IL-29 was found to be  
28 128 the major IFN expressed by stem cell-derived hepatocytes upon HCV-infection and polyI:C  
29 129 stimulation, closely mimicking the *in vivo* situation (3, 31). Viral detection and subsequent IFN-  
30 130 responses further upregulate PRR expression such as RIG-I, cGAS and STING in HLCs (3, 7).  
31 131  
32 132 Genetic variation of IL28B, another type III IFN is associated with variation in response to therapy for  
33 133 HCV-infection (32). Expression of IL28 by HLCs upon HCV-infection implicates the potential of stem  
34 134 cell-derived hepatocytes to study the influence of host genetics on viral infection. Markedly, type III  
35 135 IFNs are not expressed by HCV-infected Huh7 cells, showing the superiority of HLCs for studies on  
36 136 host-pathogen interaction (3).  
37 137  
38 138  
39 139 *2.2 Response to IFNs and the JAK/STAT pathway*  
40 140 Type I and III IFNs are released by infected hepatocytes and demonstrate autocrine and paracrine  
41 141 activity, inducing an antiviral state in the local tissue. IFN-receptors are heterodimers of  
42 142 transmembrane proteins that contain an extracellular ligand binding domain and a cytoplasmic tail.  
43 143 Hepatocytes express receptors for type I and III IFNs; IFNAR1/IFNAR2 bind type I IFNs (IFN-α and  
44 144 IFN-β) and IFNLR1/IL10-R2 bind type III IFNs (IFN-λ1/IL29, IFN-λ2/IL28A and IFN-λ3/IL28B). Upon  
45 145 binding of IFNs to their receptor, the cytoplasmic receptor-associated tyrosine kinases Janus kinase  
46 146 1 (JAK1) and Tyrosine kinase 2 (TYK2) become auto-phosphorylated. Phosphorylated JAK1 and  
47 147 TYK2 recruit and phosphorylate signal transducer and activator of transcription protein 1 (STAT1)  
48 148 and STAT2 which promotes their association with IRF9. The resulting complex of STAT1, STAT2 and  
49 149 IRF9 translocates to the nucleus where it binds to IFN-stimulated response elements (ISRE) on the  
50 150 DNA and induces expression of hundreds of ISGs (figure 1) (33). JAK/STAT signalling is negatively  
51 151 regulated by suppressor of cytokine signalling (SOCS1) which ubiquitinates phosphorylated STAT1  
52 152 and mediates its proteasomal degradation. In differentiated cells, SOCS1 is expressed at low basal  
53 153 levels but as an ISG itself becomes rapidly upregulated upon induction of the JAK/STAT pathway.  
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hESC, iPSCs and derived HLCs express all components of the IFN-signalling pathway (IFNAR<sub>1,2</sub>, STAT<sub>1,2</sub> IRF9) (24). However, hESCs and iPSCs lack the ability to respond to IFN- $\alpha$  treatment due to high basal SOCS<sub>1</sub> levels (24). Responsiveness to IFN- $\alpha$  develops early on during differentiation, correlates with downregulation of basal SOCS<sub>1</sub> levels and increases over time. D5-definitive endoderm, d15-hepatoblasts and d21-HLCs are all able to respond to IFN- $\alpha$  treatment with phosphorylation of STAT<sub>1</sub> and expression of ISGs but mature HLCs show the strongest ISG-induction levels (8, 10). The antiviral effect of the ISGs expressed by HLCs was confirmed in studies of HCV and HBV infection where IFN- $\alpha$  treatment of HLCs prior to virus infection significantly reduced viral replication and persistence (8, 10).

With emerging evidence of the importance of IL29 in hepatocyte immunity, research focused on the expression and response to type III IFNs in HLCs. Indeed, HLCs respond to IL29 treatment with phosphorylation of STAT<sub>1</sub> and expression of ISGs (IFIT<sub>1</sub>, Mx<sub>1</sub>, OAS<sub>1</sub>, ISG<sub>15</sub>, CXCL<sub>10</sub>, CXCL<sub>11</sub>, IRF<sub>9</sub>, IRF<sub>7</sub>, IRF<sub>1</sub>, IRF<sub>2</sub>) (3, 6). These findings show that pluripotent stem cell-derived hepatocytes possess functional IFN-signalling pathways which allow them to mount an innate immune response against invading pathogens that are representative of primary hepatocytes.

However, virus-induced expression of IFNs and ISGs is still reduced when compared to primary hepatocytes. One study examined the presence of HBV cccDNA, a characteristic of persistent HBV-infection, in HLCs and primary hepatocytes in the presence and absence of an inhibitor of the JAK/STAT pathway (JAKi). While cccDNA in primary hepatocytes was almost exclusively found in cells treated with JAKi, treated and also untreated HLCs harbored cccDNA. Examination of the innate immune response in HLCs revealed that although ISG-expression reflected ISG-expression in primary hepatocytes, gene expression was considerably lower in HLCs which explains why HBV cccDNA was detectable in HLCs despite an antiviral response of the cells. IL28B, IL29, IRF<sub>3</sub>, IRF<sub>9</sub> ISG<sub>15</sub>, ISG<sub>20</sub> and Mx<sub>2</sub> in d20 HLCs were only slightly upregulated compared to HBV-resistant d7 cells (7). This observation may be representative of an immature immune response in HLCs, although, more studies are needed to better understand this finding.

Studies of chronic viral infection might also benefit from HLCs having immature immune pathways. In HCV-infection for example, only a minority of HCV-positive hepatocytes is found in the *in vivo* situation (7-20%) and the infected cells seem to be of an immature phenotype (AFP+, EpCAM+) that appear in regenerating areas of the liver. While regenerating progenitor cells represent only a small proportion of cells in the liver, they contain high levels of HCV and it is assumed that they serve as a reservoir for HCV (34,35). Hence, to understand how hepatotropic viruses like HCV develop chronic infection, HLCs with reduced PRR-expression and IFN-responses could be critical.

HLCs can also be used to study immune evasion of hepatotropic viruses. Upon dengue virus (DENV) infection, ISG-expression in infected HLCs was reduced when compared to surrounding uninfected HLCs. This shows that the virus counteracts innate immune responses to evade the immune mechanisms of the infected cells (13).

### 2.3 Intrinsic Anti-Viral Mechanisms

Intrinsic immunity describes destruction of the invading pathogen performed directly by the sensing molecule. Examples of intrinsic immunity are dsRNA-activated protein kinase R (PKR) and ribonuclease L (RNaseL). Although their expression is further upregulated by IFNs which classifies them as ISGs, they are already highly expressed in uninfected cells and upon infection can become immediately activated. PKR and RNaseL are both detectable in pluripotent stem cells, although PKR has been shown to be unresponsive to polyI:C and responsiveness develops in the course of differentiation (36).



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207 3.4 *TNF-α-pathway*

208 Tumor necrosis factor-alpha (TNF-α) is a key player in liver homeostasis that regulates proliferation,  
209 survival and cell death. TNF-α signals through NF-κB and C-Jun N-terminal kinase (JNK) and cell  
210 signalling is tightly regulated as an imbalance of these pathways results in liver diseases such as  
211 cancer development or hepatic failure (37). TNF-α has been shown to play a prominent role in the  
212 pathogenesis of chronic HCV- and HBV infection (37).

213  
214 TNF-α signalling through NF-κB promotes cell survival and induces expression of various  
215 proinflammatory cytokines while JNK-signalling induces apoptosis (14). Binding of soluble TNF-α to  
216 its receptor TNFR1 on the cell surface induces homotrimer formation of TNFR1. Adaptor protein  
217 TNFR-associated death domain (TRADD) is recruited and binds to the cytosolic region of the  
218 receptor. Upon receptor binding, TRADD interacts with the downstream signalling molecules TNF-α  
219 receptor-associated factor 2 (TRAF2) and receptor-interacting protein kinase (RIP). The complex of  
220 TRADD, TRAF2 and RIP can activate both, NFκB- and JNK-signalling, leading to either cell survival  
221 or death. NFκB is a heterodimer of the two subunits p65 and p50. In the quiescent state, IκB binds to  
222 NFκB, thereby inhibiting NFκB and preventing its nuclear localisation. TNF-α signalling activates the  
223 IκB-kinase (IKK) complex, consisting of the kinase subunits IKKα and IKKβ and the regulatory  
224 subunit IKKγ. Phosphorylation of IKKβ activates the complex and induces IκBα degradation via Ser  
225 32 and 36 phosphorylation which marks the protein for ubiquitination on Lys 21 and 22 and  
226 subsequent degradation by the proteasome 26S complex (38). Released from their association with  
227 IκB, NFκB heterodimers translocate to the nucleus where they transactivate pro-inflammatory  
228 cytokine expression, such as TNF-α, CXCL10, CXCL11 and IL-8 as well as pro-survival genes such as  
229 caspase-8 inhibitor c-FLIP and Gadd35β.

230  
231 HLCs release TNF-α in response to infection and respond to stimulation with recombinant TNF-α.  
232 Hence, they represent a valuable opportunity to study TNF-α signalling in infection, proliferation  
233 and carcinogenesis. This will also help to better understand the mechanisms that regulate the  
234 interplay between TNF-α induced NFκB- and JNK-signalling and thereby the regulation of cell  
235 survival and cell death. Studies in this field had been hampered by the lack of an appropriate cell  
236 system. Although hepatoma cell lines such as Huh7 cells greatly contributed to our current  
237 understanding of cell cycle regulation, these cell lines represent cancer tissue. Deregulated cell  
238 death/survival pathways are the main feature of hepatoma cell lines, making these cells an  
239 unreliable model. The high survival capacity of Huh7 cells, for example, is promoted by high basal  
240 NFκB levels. Understanding the mechanisms that regulate cell death and survival would be of  
241 therapeutic value and could lead to the identification of therapies that prevent cell death in the  
242 context of liver injury or suppress carcinogenesis and HLCs could be of great value in these studies.  
243 Viral proteins such as dengue virus (DENV) NS1 can directly activate NFκB-signalling for TNF-α  
244 production in HLCs (13). DENV infection of hESC- and iPSC-HLC activates NFκB-signalling as  
245 determined by phosphorylation of the NFκB subunits p65/p50. As phosphorylation of p65/p50 was  
246 not limited to infected cells, NFκB activation does not only seem to be a result of direct virus-sensing  
247 but is also induced by the paracrine mechanism of TNF-α released from infected cells. NFκB-  
248 activation resulted in synthesis and release of proinflammatory cytokines such as CXCL10, CXCL11,  
249 IL6 and to a smaller extent IL8 and TNF-α and downregulation of normal hepatocyte-genes such as  
250 albumin, E-cadherin, coagulation factor V and proteins of the complement system such as F5.  
251 Significant downregulation of albumin and F5 is also seen in DENV-infected primary hepatocytes  
252 (13).

253  
254  
255 2.5 *TLR expression and downstream signalling pathways*

256 Toll-like receptors (TLR) are central in pathogen sensing and activation of immune responses. The  
257 TLR-response in hepatocytes is tightly regulated *in vivo* which is crucial in an organ that is constantly  
258 exposed to various antigens, including pathogens coming from the gut. In this microenvironment,

the hepatocyte has to stay alert to invading pathogens and initiate appropriate immune responses while TLR over-activation is associated with pathogenesis of chronic inflammation and disease. In healthy hepatocytes mRNA for all TLRs is only marginally expressed but upon exposure to certain stimuli, specific TLRs can be upregulated (39). Further, TLR-signalling and deregulation influences carcinogenesis. It is therefore not surprising that TLR-expression in hepatoma cell lines is modified. TLR3 and 7 appears to be under expressed in hepatoma cell lines which makes these cells unsuitable for studies for TLR-responses to viruses and might explain why the role of TLRs in hepatocytes is often underestimated (40).

PAMP recognition by a specific TLR induces signalling through NF $\kappa$ B, leading to the expression of numerous host defense genes. Nine functional TLRs are known in humans (TLR1-9). TLR3 (which detects dsRNA), TLR7 (which detects ssRNA) and TLR9 (which detects non-methylated DNA CpG-motifs) are located within the endosomes of hepatocytes while TLR2, TLR4 and TLR5 are localized on the cell membrane. Binding of a ligand to its receptor triggers signalling via MyD88 for TLR2, TLR4, TLR5, TLR7/8 and TLR9 and TRIF for TLR3. MyD88 is anchored to the cytoplasmic TIR-domain of the TLR by its own TIR-domain. Activated MyD88 recruits interleukin 1 receptor associated kinase (IRAK), leading to IRAK auto-phosphorylation. Phosphorylated IRAK detaches from MyD88 and activates TRAF6 which then activates the IKK-complex resulting in NF $\kappa$ B translocation to the nucleus (see TNF-signalling) and expression of pro-inflammatory cytokines such as TNF- $\alpha$ , CXCL10, CXCL11 and IL-8 (41). While in professional immune cells such as plasmacytoid dendritic cells, TLR7/8 and TLR9 also signal via IRF7, leading to IFN- $\alpha$  and IL29 expression; data as to whether hepatocytes possess the IRF7 arm of TLR-signalling are conflicting. A study in HepG2 cells reported absence of IRF7 signalling upon TLR7 stimulation (42). However, as mentioned before, hepatoma cell lines are of limited value in the study of TLR-responses. HLCs would be of great benefit if expression of TLR-proteins and components of the signalling pathways are expressed at the end of the differentiation procedure.

TLR-expression by pluripotent stem cell-derived somatic cells has been studied but with the exception of Sakurai and colleagues who detected TLR3 mRNA expression in iPSC-derived hepatocytes, none of these examined HLCs (6). Földes and colleagues investigated responsiveness of hESC, hESC-derived endothelial cells (hESC-EC) and primary human endothelial cells to ligands for TLR1-9 (43). In contrast to primary cells, neither hESC nor hESC-ECs responded to TLR-agonists with the exception of TLR5 which was already expressed in hESC. This lack was attributed to the absence of TLR-protein expression as stimulants that likewise signal through NF $\kappa$ B but act through different receptors (TNF- $\alpha$ , IL-1 $\beta$ , IFN- $\gamma$ ) induced IL8 expression in all three cell lines. qPCR-analysis revealed very low expression of TLR mRNA in hESC-EC and especially hESCs. Amounts of NF $\kappa$ B protein were comparable between hESC, hES-ECs and primary endothelial cells and increased only marginally in the course of differentiation. NF $\kappa$ B-signalling could be induced upon TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$  stimulation. This finding shows that hESC-derived ECs do not express TLRs although inflammatory signalling pathways are present and functional in hESCs and hESC-ECs. Markedly, *in vivo* conditioning of hESC-ECs by injection into nude mice for 21 days did not establish TLR-expression (44).

In contrast, two studies on hESC-derived fibroblasts and keratinocytes reported expression of TLR2 and 4 and responsiveness to Gram-negative bacteria (45, 46). However, both TLRs were already expressed in hESC and responsiveness to bacterial challenge in hESC-fibroblasts appeared to be less specific compared to primary cells (45).

The discrepancies amongst published data emphasis the importance of studying TLR-expression in stem-cell derived somatic cells more closely. Inducibility of TLR-expression and signalling in HLCs is of particular interest as tightly regulated hepatic TLR-responses *in vivo* are critical to maintain the balance between immune activation and tolerance in the liver. In primary hepatocytes, virus-induced downregulation of TLR7 has been reported, further emphasising the need to study TLR-responses in HLCs (47).



We are currently examining expression and inducibility of endosomal TLRs in HLCs from different backgrounds. As TLR responses develop late in the embryo and are again repressed after birth, deriving hepatocytes with functional TLR-responses from hESCs can be challenging. iPSCs might be of advantage here, considering their somatic cell origin. Indeed, expression and responsiveness of TLR2 and 4 has been detected in iPSC-derived endothelial cells while absence of TLR-expression in hESC-ECs was confirmed in this study (44).

2.6 Acute Phase Protein (APP) Production

The acute phase response describes a systemic immune response against sepsis where hepatocytes secrete defensive proteins into the circulation. The acute phase is initiated by inflammatory cytokines including IL-1 $\beta$ , TNF- $\alpha$  and IL-6 which become upregulated upon inflammation. IL-6 induces secretion of C-reactive protein,  $\alpha$ 1-antichymotrypsin, serum amyloid A and fibrinogen while secretion of albumin, transferrin and fibronectin becomes downregulated. However APP production by pluripotent stem cell-derived hepatocytes has not been studied extensively. Irudayam and colleagues analysed gene expression of acute phase proteins during hepatic differentiation of hESC and found a 10 to 1,000 fold upregulation of genes coding for acute phase proteins, coagulation factors and several members of the acute phase response such as serum amyloid A1,  $\alpha$ 1-antitrypsin and  $\alpha$ 2-macroglobulin in HLCs compared to definitive endoderm (9). However, only basal expression of acute phase proteins was examined and the results were not compared to primary hepatocytes. LPS- or IL6- induction of the acute phase response in HLCs has not been tested. It is therefore difficult to determine whether the acute phase response seen in HLCs is fully representative of the *in vivo* situation meriting further investigation.

3.0 Conclusion

*In vitro* differentiated cells such as HLCs, endothelial cells, cardiomyocytes, smooth muscle cells and osteoblasts differ from their *in vivo* counterparts in numerous ways. This includes the innate immune response. This immature phenotype is characterised by reduced levels of ISG-expression upon infection or stimulation with TLR-ligands. Production of IFNs upon infection and ISG-expression are the major innate immune mechanisms in hepatocytes. These pathways have been shown to be expressed in HLCs. However, other pathways also play important roles in the clearance of infection. Studies on HBV-infection of primary hepatocytes from different donors reported huge variation in virus susceptibility and in some cases even suppression of the IFN-based immune responses could not reconstitute virus replication (7). This shows that other pathways of the innate immune response play a role in susceptibility to disease, highlighting that IFN pathways in HLCs alone are not sufficient to study inter-individual differences in virus susceptibility. This finding also demonstrates that it is necessary to improve differentiation protocols to deliver intact innate immune pathways in HLCs, to allow further mechanistic studies. One strategy to improve maturation of innate immune pathways could be the addition of immunostimulatory cytokines to the differentiation medium as is standard for the differentiation of immune cells such as natural killer cells and dendritic cells. Innate immune pathways in these cells are more developed than in tissue cells.

The lack of certain pathways of the innate immune response such as TLR-signalling might have important implications for tissue regeneration, drug-testing and host-pathogen studies. For studies on host-pathogen interaction, mature immune signalling will be essential while for regenerative medicine, immature immune signalling can be beneficial in certain cases, including transplant acceptance. Pathogens are generally detected by more than one sensing molecule, for example TLR4 and the cytoplasmic receptor NOD-I both sense Gram-negative bacteria. In contrast to NOD-I, TLR4 is also associated with vascular inflammation in endothelial cells and hESC-ECs that do not express TLR4 sense Gram-negative bacteria by NOD-I and mount comparable levels of antiviral cytokines. hESC-ECs that lack TLR-responses might therefore be a promising target for transplantation into patients with atherosclerosis (44). The same would apply to situations where tissue or organ damage is a result of excessive immune responses.

In conclusion, pluripotent stem cell-derived HLCs are superior to hepatoma cell lines for mimicking hepatic innate immunity. However, innate immunity is compromised in HLCs when compared to primary hepatocytes (figure 2) necessitating future improvements in cellular differentiation protocols from PSCs.

#### 4.0 Competing Interests

Professor David C Hay is a founder, director and shareholder in Stemnovate Limited.

#### 5.0 Acknowledgements

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**Figure 1: Innate immune signalling pathways in hepatocytes.** Hepatocytes possess sensors for DNA- and RNA viruses in the cytoplasm and in endosomes which, upon activation, induce the expression of IFNs and inflammatory cytokines. IFNs act in an autocrine and paracrine manner turning the cells into an antiviral state. Upon receptor binding of TNF- $\alpha$  signalling via NF $\kappa$ B leads to expression of inflammatory cytokines and chemokines.

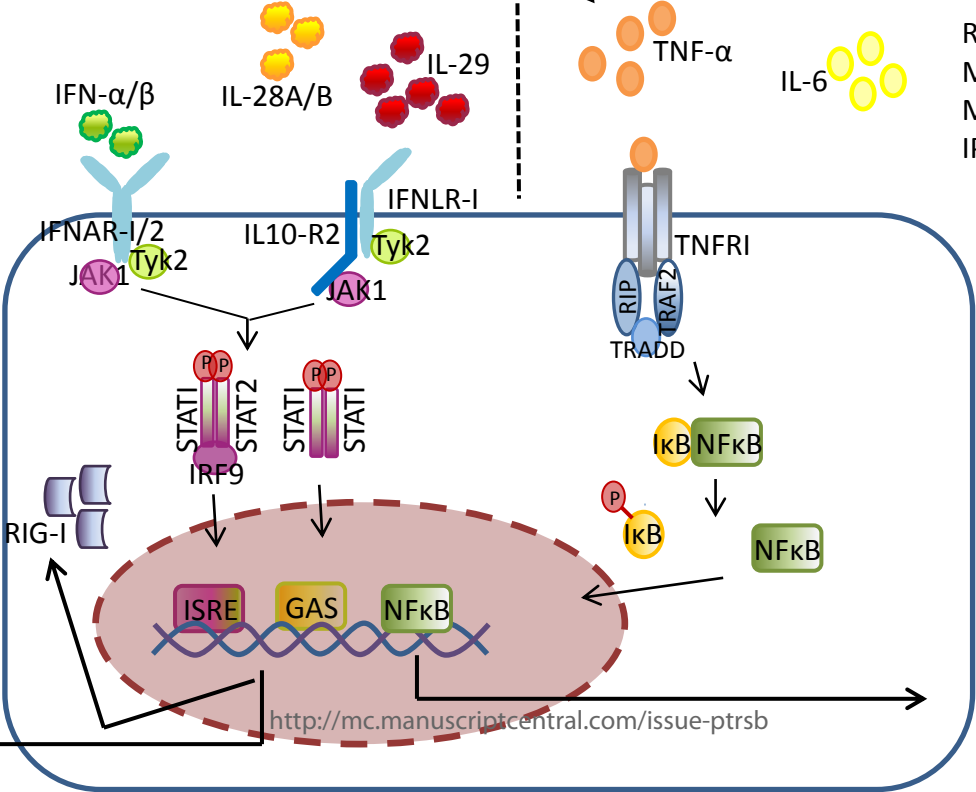
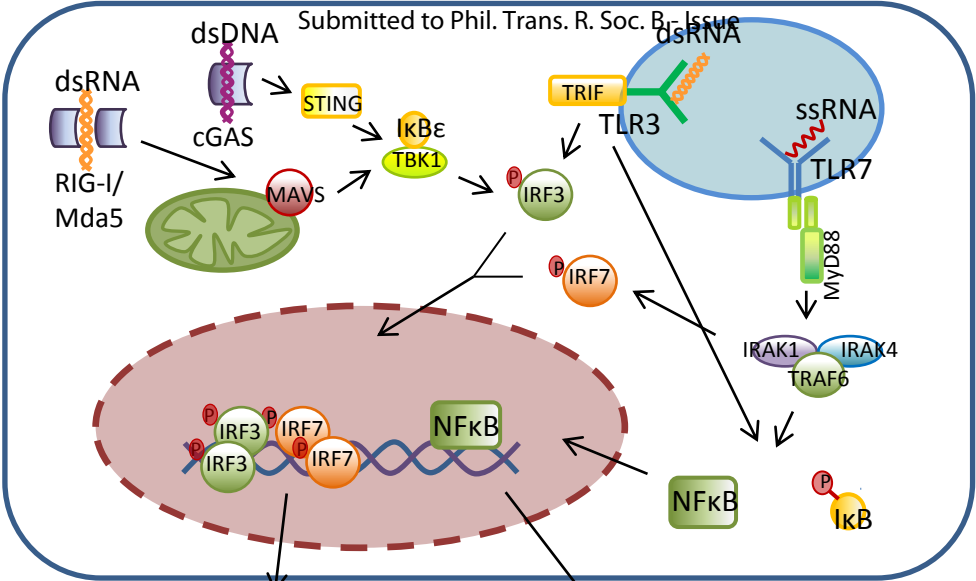
Abbreviations: ds – double stranded, RIG – retinoic acid inducible gene, Mda – melanoma differentiation associated gene, MAVS - mitochondrial antiviral signalling protein, TBK – tank-binding kinase, IRF – interferon regulatory factor, TLR – toll-like receptor, IRAK – interleukin 1 receptor associated kinase, TRAF - TNF- $\alpha$  receptor-associated factor, IFN – interferon, STAT – signal transducer and activator of transcription protein, ISRE – IFN-stimulated response element, TNF- $\alpha$  – tumor necrosis factor alpha, TNFR – tumor necrosis factor receptor, NF $\kappa$ B - nuclear factor kappa-light-chain-enhancer of activated B cells, IL –interleukin, CXCL10 - C-X-C motif chemokine

**Figure 2: Comparison of innate immune pathways in pluripotent stem cells, stem cell-derived hepatocytes and primary hepatocytes.** Pluripotent stem cells and primary hepatocytes employ fundamentally different mechanisms to defend themselves against invading pathogens. Innate immune pathways in HLCs resemble primary hepatocytes, however, reduced levels of ISG-expression and possible absence of TLR-responses represent immaturity of immune signalling pathways.

Abbreviations: HLC –hepatocyte-like cells, ISG – interferon stimulated genes, PRR – pattern recognition receptor, SOCS - suppressor of cytokine signalling, NF $\kappa$ B - nuclear factor kappa-light-chain-enhancer of activated B cells, RIG – retinoic acid inducible gene, Mda – melanoma differentiation associated gene, IFN – interferon, ISG- interferon stimulated genes, IL – interleukin, TNF- $\alpha$  – tumor necrosis factor alpha CXCL - C-X-C motif chemokine, TLR – toll-like receptor.



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### Pluripotent stem cells

- Cytosolic PRRs and signalling components downregulated → only marginal IFN-expression
- High basal SOCS1 levels prevent IFN-induced ISG-expression
- NFκB pathway present
- RNAi as antiviral mechanism

### Hepatocyte-like cells

- RIG-I/Mda5 detect dsRNA → IFN-expression
- Viral DNA-detection via cGAS-STING pathway → IFNs
- IFN-pathway present but weaker ISG-induction than PHH
- IL29 as dominant IFN
- NFκB pathway present
- Stimulation with TNF-α induces inflammatory gene expression
- TLR mRNA undetectable in healthy cells; possible upregulation upon stimulation unknown

### Primary human hepatocytes

- RIG-I/Mda5 detect dsRNA → IFN expression
- Viral DNA-detection via cGAS-STING pathway → IFNs
- IFN-response induces ISG-expression
- IL29 as dominant IFN
- NFκB pathway present
- TNF-α induces inflammatory gene expression (CXCL10, CXCL11, TNF-α, IL8)
- Low-level TLR mRNA expression; upregulation and protein expression in inflammatory environment